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STUDIES ON THE NEURAMINIDASES OF INFLUENZA VIRUS

III. STIMULATION OF ACTIVITY BY BIVALENT CATIONS

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SUMMARY

Bivalent cations (Ca^{2+}, Mn^{2+} and Mg^{2+}) have been shown to stimulate the activity of the purified neuraminidases (*N*-acetylneuraminate glycohydrolase, EC 3.2.1.18) from influenza virus although an absolute requirement could not be demonstrated. This stimulation is dependent upon the enzyme and is independent of the substrate and the nature of the linkage of the *N*-acetylneuraminic acid. The addition of Ca^{2+} increases the v_{max} but has no effect on the K_m of the reaction.

INTRODUCTION

Bivalent cations have been shown to be necessary for the activity of neuraminidases (*N*-acetylneuraminate glycohydrolase, EC 3.2.1.18) derived from certain sources. WARREN AND SPEARING[1] reported that Ca^{2+} stimulated a mammalian neuraminidase and that 5 mM EDTA inhibited the enzyme. MOHR AND SCHRAMM[2] showed that 10 mM $CaCl_2$ or $MnCl_2$ was required for maximal activity of the purified neuraminidase from *Vibrio cholerae*. ROSENBERG, BENNIE AND CHARGAFF[3] corroborated this and demonstrated that if the *V. cholerae* neuraminidase was treated with EDTA and dialyzed, activity was lost which could be restored with 4 mM Ca^{2+}. Mn^{2+} and Co^{2+} were almost as effective as Ca^{2+} and Mg^{2+} restored only 29% of the activity. MAYRON *et al.*[4] and RAFELSON, SCHNEIR AND WILSON[5] reported that the neuraminidases of influenza virus were not stimulated by Ca^{2+} although the enzymes were inhibited by EDTA. Addition of Ca^{2+} or Mn^{2+} reversed the EDTA inhibition. Removal

Abbreviations: 3-NAN-LAC, *N*-acetylneuraminyl-(2 → 3)-β-D-galactopyranosyl-(1 → 4)-D-glucopyranose; 6-NAN-LAC, *N*-acetylneuraminyl-(2 → 6)-β-D-galactopyranosyl-(1 → 4)-D-glucopyranose; NAN-LAC, refers to *N*-acetylneuraminyl-lactose containing among other components both 3-NAN-LAC and 6-NAN-LAC; NANA, *N*-acetylneuraminic acid.

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of the EDTA by dialysis also relieved the inhibition, suggesting that EDTA had to be present in the incubation medium to produce the inhibition. The present study shows that a bivalent cation is in fact required for maximal enzyme activity although an absolute requirement could not be demonstrated. Our earlier inability to demonstrate stimulation by bivalent cations was due most probably to adventitious cations in both the substrates and enzyme preparations used.

MATERIALS AND METHODS

Isolation of neuraminidases

The enzymes were isolated from chymotrypsin-treated PR8 and Jap 305 strains of influenza virus as described by RAFELSON, GOLD AND PRIEDE[6] with the exceptions that the dimensions of the Sephadex G-200 column bed were 15 mm × 750 mm and 5-ml fractions were collected at a flow rate of 5 ml/h.

Preparation of substrates

3-NAN-LAC and 6-NAN-LAC were isolated from bovine colostrum by the method of SCHNEIR AND RAFELSON[7]. Colominic acid was isolated respectively by the method of MCGUIRE AND BINKLEY[8].

Assay of enzymes

Neuraminidase

The standard assay mixture consisted of enzyme solution, 0.1 ml of 3-NAN-LAC solution (4 mg/ml) and 0.1 M Tris-maleate buffer (pH 6.5) for the Jap 305 enzyme and pH 7.0 for the PR8 enzyme to a final volume of 0.5 ml. The mixture was incubated at 37° and appropriate samples (0.1–0.2 ml) were removed at suitable time periods (10 and 20 min) for analysis of the liberated NANA by the thiobarbituric acid procedure of WARREN[9]. The enzyme reaction is stopped by the addition of the metaperiodate reagent in the assay for NANA. A unit of enzyme activity is defined as the amount of enzyme that releases 1 μmole of NANA per min from 3-NAN-LAC under the above conditions.

Protein determinations

Protein was determined by the method of LOWRY *et al.*[10] using crystalline bovine albumin as the standard.

Chymotrypsin

Chymotrypsin was assayed with *N*-benzoyl-L-tyrosine ethyl ester as the substrate[11].

RESULTS

*Requirement for bivalent cation**

The first direct evidence that a bivalent cation might be required for maximal neuraminidase activity came from experiments in which there was a low recovery of enzyme activity from the Sephadex column. A representative Sephadex column fractionation is shown in Fig. 1 in which only 28% of the neuraminidase activity

* Although only data for the Jap 305 enzyme are reported here, identical results were obtained for the enzymes from the PR8, Great Lakes and Maryland B strains.

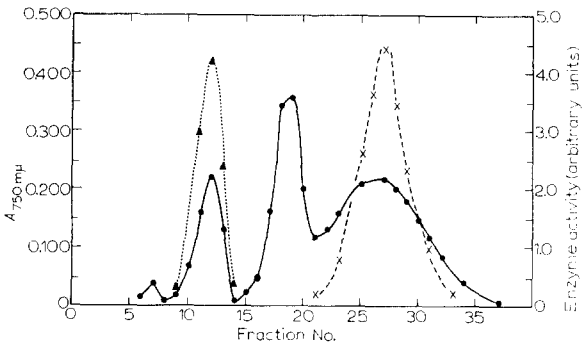


Fig. 1. Fractionation of chymotrypsin-treated Jap 305 Virus on Sephadex G-200. ●—●, absorbance at 750 mμ (LOWRY); ▲· · · ·▲, neuraminidase activity; × — — — ×, chymotrypsin activity.

TABLE I

NEURAMINIDASE ACTIVITY OF MIXED SEPHADEX FRACTIONS

*Fractions assayed**	*Enzyme units*	*% Stimulation*
11–13	10.8	—
11–13 + 1–8**	10.7	0
11–13 + 15–25**	10.2	0
11–13 + 26–35**	10.5	0
11–13 + 36–50**	39.6	266
11–13 + 36–50*** (dialyzed)	10.6	0
11–13 + 36–50**** (heated)	39.2	263

* Fractions from Fig. 1, 36–50 not shown.

** Fractions combined, lyophilized and reconstituted in 2.0 ml of buffer; 0.1 ml of each added to 0.1 ml of Fractions 11–13 and the mixture reassayed for neuraminidase activity.

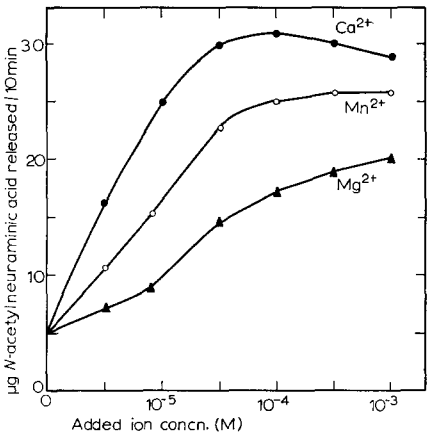
*** Dialyzed 16 h against Tris–maleate buffer.

**** Heated for 15 min at 100°.

added to the column was recovered in the effluent fractions. In order to test for the possible removal of a 'cofactor' from the enzyme, various of the column fractions (1–8, 15–25, 26–35, 36–50) were separately combined, lyophilized, dissolved in 2.0 ml of buffer and 0.1 ml of each added separately to aliquots of pooled enzyme Fractions 11–13. Reassay of pooled Fractions 11–13 (Table I) showed that addition of the reconstituted Fractions 36–50 increased the neuraminidase activity some 266%, Fractions 1–8, 15–25 and 26–35 being without measurable effect. As shown in the last two experiments in Table I, the stimulatory substance(s) in Fractions 36–50 was dialyzable and stable to heating at 100° for 15 min.

Preliminary spectrographic analysis indicated that Fractions 36–50 contained significant amounts of Ca^{2+}, Mg^{2+} and Mn^{2+}. The cofactor activity of Fractions 36–50 could be replaced in descending order of effectiveness by the addition of Ca^{2+}, Mn^{2+} or Mg^{2+} (Fig. 2).

Fig. 3 shows that the optimal pH for the action of the Jap 305 neuraminidase on three substrates (3-NAN-LAC, 6-NAN-LAC and colominic acid) was unaffected by the addition of 10^{-4} M Ca^{2+}.



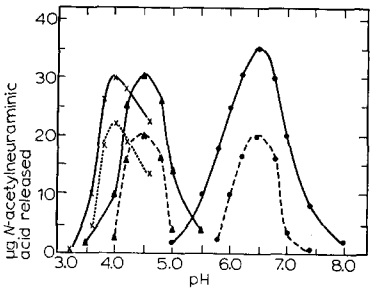
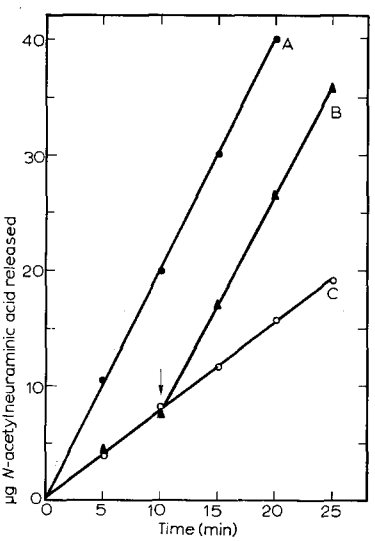


Fig. 2. Effects of added Ca^{2+}, Mn^{2+} and Mg^{2+} on neuraminidase activity.

Fig. 3. pH optima of Jap 305 neuraminidase for two isomers of N-acetylneuraminic acid and colominic acid in presence and absence of 10^{-4} M Ca^{2+}, ●—●, 3-NAN-LAC + Ca^{2+}; ●— — —●, 3-NAN-LAC; ▲—▲, 6-NAN-LAC + Ca^{2+}; ▲— — —▲, 6-NAN-LAC: ×—×, colominic acid + Ca^{2+}; × · · · · · ×, coliminic acid.



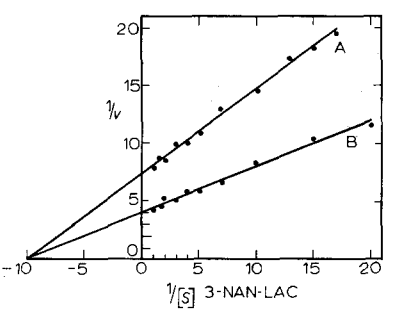


Fig. 4. Effect of delayed addition of Ca^{2+} on neuraminidase activity. Curve A, 10^{-4} M $CaCl_2$ added at zero time; Curve B, 10^{-4} M $CaCl_2$ added at 10 min; Curve C, no $CaCl_2$ added.

Fig. 5. Effect of Ca^{2+} on neuraminidase activity with varying concentrations of substrate. Curve A, 3-NAN-LAC; Curve B, 3-NAN-LAC + 10^{-4} M Ca^{2+}.

In the experiments reported above the bivalent ion was added prior to or at the same time as the addition of the substrate. That this is not necessary to obtain the stimulatory effect is shown in Fig. 4. In this experiment the reaction with 3-NAN-LAC was allowed to proceed for 10 min in the absence of Ca^{2+}. At this time 10^{-4} M Ca^{2+} was added to an aliquot of this mixture and the reaction followed for an additional

TABLE II

EFFECT OF DIALYSIS ON NEURAMINIDASE ACTIVITY

Sample	*Enzyme units**	
	Without Ca^{2+}	*With* 10^{-4} *M* Ca^{2+}
Original enzyme	2.00	4.36
Stored 7 days at 4°	1.95	4.25
Dialyzed 7 days against buffer containing 10^{-3} M EDTA and redialyzed against buffer alone	0.40	4.20

* Assayed with 3-NAN-LAC.

15 min (Curve B). It may be seen that the rate of the reaction increased and approximated very closely the rate of the reaction in which 10^{-4} M Ca^{2+} was present from zero time (Curve A). The rate of the reaction over the 10–25-min period in an aliquot of the incubation mixture to which Ca^{2+} was not added was unchanged over that of the first 10 min.

The stimulatory effect of Ca^{2+} can be obtained over a relatively wide range of concentrations of 3-NAN-LAC. This is shown in Fig. 5 which is a double-reciprocal plot showing the rate of 3-NAN-LAC cleavage in the absence and presence of added 10^{-4} M Ca^{2+}. Although there is a change in the v_{max}, there appears to be no effect on the K_m.

Failure to establish absolute requirement for bivalent cation

Extensive attempts were made to remove bivalent cations from both the enzyme preparations and the substrates employed. For example, the enzyme preparations were dialyzed against 100 vol. of Tris–maleate buffer containing 10^{-3} M EDTA for 7 days at 4°, the buffer being changed each 24 h. The EDTA-containing samples were redialyzed for 25 h against buffer alone to remove the EDTA prior to assay for

TABLE III

EFFECTS OF VARIATION IN SUBSTRATE CONCENTRATIONS ON THE STIMULATION OF NEURAMINIDASE BY Ca^{2+}

Substrate in incubation tube (μg)	*% Stimulation due to* 10^{-4} *M* Ca^{2+}		
	Colominic acid	*NAN-LAC*	*3-NAN-LAC**
50	40	76	242
100	20	33	245
200	10	12	248
300	0	3	252
400	0	0	250
600	0	0	246
800	0	0	233
1000	0	0	246

* Purified by passage through column of Dowex 50-X8 (Na^+ form).

TABLE IV

Ca^{2+}, Mn^{2+} AND Mg^{2+} CONTENT OF SUBSTRATES USED

Compound	*% Ca^{2+} + Mn^{2+} + Mg^{2+}*
3-NAN-LAC*	0.1
3-NAN-LAC	1
NAN-LAC	2
Colominic acid	2–3

* Passed through Dowex 50-X8 column (Na^+ form).

enzyme activity. This experiment is shown in Table II. The prolonged dialysis reduced the activity of the enzyme to 20% of the original level. Similar experiments were repeated with a rigorous attempt being made to exclude bivalent ions from all solutions including the substrate 3-NAN-LAC which was passed trough a column of Dowex 50-X8 in the sodium form prior to use. The results were identical to those reported above and we have not as yet been able to establish an absolute requirement for a bivalent cation.

That the substrates as usually prepared contain bivalent cations is known from direct analysis[7] and from the observation that the degree of bivalent ion stimulation decreased with increasing concentrations of certain substrates (Table III). The Ca^{2+} stimulation was completely removed by increasing concentrations of colominic acid and NAN-LAC, whereas increasing concentrations of specially prepared 3-NAN-LAC were without effect. Table IV shows the content of Ca^{2+} + Mn^{2+} + Mg^{2+} in the substrates used as determined by spectrographic analysis. It seems clear that significant amounts of bivalent cations are added to the enzyme assay medium when NAN-LAC, colominic acid and the usual 3-NAN-LAC preparation are used. For example, if it is assumed that all the cation is Ca^{2+}, 400 μg of NAN-LAC would provide 8 μg of Ca^{2+} or a concentration of $4 \cdot 10^{-4}$ M in the enzyme assay medium. This concentration, as shown in Fig. 2, markedly stimulated the enzymes.

DISCUSSION

Contrary to earlier published reports from this laboratory[4,5] bivalent cations have been found to stimulate the activity of the neuraminidases from influenza virus. The stimulation was dependent upon the enzyme and not upon the substrate and the nature of the linkage of NANA. This was shown through the use of three substrates, 3-NAN-LAC, 6-NAN-LAC and colominic acid which respectively have NANA linked in a $2 \rightarrow 3$, a $2 \rightarrow 6$ and a $2 \rightarrow 8$ glycosidic bond. The inability to demonstrate an absolute requirement for a bivalent cation may be explained on the basis that the enzymes have some activity in the absence of a bivalent cation or that the enzymes as isolated contain some bound metal ions. If the enzymes do indeed contain divalent cations, one must conclude that they are very tightly bound, since dialysis for 7 days against EDTA apparently failed to remove them. The problem of adventitious metal ions is both difficult to assess and to control, especially with the substrates which appear to bind bivalent cations very strongly.

The mechanism of stimulation of neuraminidase activity by bivalent cations is not known. It was observed that the v_{max} but not the K_m was altered. Whether the

metal ion acts on the enzyme or substrate or both cannot be answered by the present experiments and must await detailed kinetic studies.

Our earlier inability to demonstrate metal ion stimulation is most probably related to the purity of both enzyme and substrate preparations used. It is likely that the present Sephadex columns are more effective than earlier ones in removing bivalent cations from the enzymes. It is clear that substrates prepared in the usual way contain sufficient amount of $Ca^{2+} + Mn^{2+} + Mg^{2+}$ to obscure a requirement for a bivalent cation.

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